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### Java project on periodontal disease. Periodontal condition in relation to vitamin C, systemic conditions and tooth loss

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## Chapter 4

Effect of Vitamin C Supplementation in an Untreated Periodontitis Population.

An observational study

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**Abstract**

**Objective:** To assess in a periodontally diseased population deprived from regular dental care and having poor dietary conditions, the effect of vitamin C supplementation in combination with flavonoids on plasma levels of vitamin C, HbA1c, CRP and the subgingival microbiological load in periodontal pockets.

**Material & Methods:** The study population consisted of 98 subjects from the Purbasari tea estate on West Java, Indonesia. For this supplementation study all subjects were instructed to consume one tablet a day containing 200 mg Ester C<sup>®</sup> calcium ascorbate, 25 mg calcium threonate and 100 mg citrus flavonoids (vitamin C/Ca/FI) for 90 days. The following parameters were evaluated: plasma vitamin C, vitamin D, HbA1c and CRP, the presence of putative periodontopathic bacteria and viruses.

**Results:** The mean plasma vitamin C value at baseline was 5.19 mg/l and included 44 subjects (45%) who showed plasma vitamin C values <4.0 mg/l. After supplementation, in all individual subjects the plasma vitamin C values rose to levels above 4.0 mg/l with a mean of 12.1 mg/l, ranging from 4.1 to 21.2 mg/l. After supplementation all subjects had lower HbA1c levels than before, interestingly 41% of the subjects in the pre-diabetic group changed to the normal group. CRP levels were also lower in all individuals after supplementation than before, decreasing from 2.16 to 0.94 mg/l. For all studied bacteria and Epstein Barr virus a significant decrease in their quantity was found.

**Conclusion:** in populations with poor dietary conditions emphasis should be paid by the authorities to improve the fruit intake.

## Introduction

The importance of fruits, containing relatively large amounts of vitamin C, for human health has been known for quite some time due to its use in the prevention and treatment of scurvy (Luca & Norum 2011). At present, vitamin C has been shown not only to be related to scurvy but also to a number of other conditions and diseases. In addition to vitamin C, fruits contain a variety of flavonoids with health related properties (Landete 2012).

Research in the diabetes field tends to show lower levels of plasma vitamin C in subjects with diabetes type 2 as compared to healthy controls (Sinclair et al. 1994 and discussed by Will et al. 1999). Interestingly, in adults with type 2 diabetes reduced vitamin C content of mononuclear leukocytes (Cunningham et al. 1991) and lymphocytes has been reported (Yamada et al. 2004). In addition, a more recent prospective study showed that higher plasma vitamin C levels were associated with a substantially decreased risk of type 2 diabetes development (Harding et al. 2008). Apart from vitamin C, it has been reported also that diets rich in flavonoids are related also with a lower incidence of diabetes (Zamora-Ros et al. 2013).

Cardiovascular research revealed in many studies a positive association between high-sensitivity C-reactive protein (CRP), a marker of inflammation, and cardiovascular disease (Musunuru et al. 2008). It has also been shown that plasma vitamin C levels are inversely associated with CRP (Wannamethee et al. 2006). Like vitamin C, flavonoids are found to be inversely related to CRP plasma values (Filiberto et al. 2013) and high intakes of dietary flavonoids are associated with decreased cardiovascular disease mortality and risk factors (Toh et al. 2013).

Another chronic disease frequently associated with vitamin C, concerns periodontal diseases i.e. diseases of the supporting soft- and hard tissues of the teeth initiated by putative periodontopathic microorganisms. Well known is the relationship between necrotizing ulcerative gingivitis (NUG) and vitamin C deficiency (Melnick et al. 1988). In addition, it has been shown that plasma vitamin C levels are inversely related to the severity of periodontitis (Amarasena et al. 2005, Amaliya et al. 2007, Chapple et al. 2007). The limited research available on the effect of vitamin C supplementation in periodontal diseases, either by diet or supplements, showed that gingival bleeding may decrease (Leggott et al. 1986, Jacob et al. 1987, Staudte et al. 2005). This could be explained by a reduction in periodontopathic microorganisms as a result of an improved host response. However, it does not result in a reduction of the periodontal lesion (Leggott et al. 1991). Little research is available concerning a possible relationship between flavonoids and periodontal disease. In one

epidemiological study a significant inverse dose-response relationship between the intake of isoflavones and the prevalence of self reported periodontal disease was observed (Tanaka et al. 2008).

In 2005 plasma vitamin C levels were assessed in an Indonesian population that had been part of an investigation of the natural development of periodontitis. In this cohort, deprived from regular dental care and aged at that time 33-43 years, an inverse relationship was found between plasma vitamin C levels and the amount of periodontal breakdown (Amaliya et al. 2007). Also, 28.5% of the study population was depleted/deficient of vitamin C, i.e. plasma values <4.0 mg/l. The purpose of the present study was twofold. Firstly, to investigate whether the subjects that were deficient/depleted of vitamin C in the previous study (Amaliya et al. 2007) had indeed a reduced capacity to absorb vitamin C. Secondly, to evaluate in this periodontally diseased population whether supplementation of vitamin C in combination with flavonoids results in increased levels of plasma vitamin C, decreased plasma levels of HbA1c and CRP and whether it has an effect on the subgingival microbiological load in periodontal pockets.

### **Materials and Methods**

In 2005 plasma vitamin C levels were assessed in 123 subjects of the original Indonesian study population (Amaliya et al. 2007). In 2011, the same 123 subjects were asked to participate in the present study. Prior to the start of the study, subjects were informed in detail about the objectives of the investigation. The study was approved by the Ethics Committee of the Hasan Sadikin Hospital Bandung-West Java, Indonesia. This vitamin C supplementation study is part of a larger study in which the amount of periodontal bone loss was studied in relation to the levels of plasma vitamin C, vitamin D, HbA1c and CRP, the Hp phenotype, the presence of putative periodontopathic bacteria and viruses, dietary habits and anthropometrics (Amaliya et al. 2014).

It was the intention of the authors to perform a placebo controlled clinical trial. However no permission was granted by the Ethic Committee since this population participated already since 1987 in a longitudinal prospective study without any treatment. Also it had already been shown that almost one third of the population was depleted/deficient of vitamin C. Therefore, all subjects in this study had to be provided with vitamin C supplementation resulting in a prospective cohort study.

### *Clinical procedures*

At baseline, the 98 subjects that could be retrieved and who volunteered to participate in the study, signed the informed consent. Subsequently age, gender and smoker status were recorded. Non-smokers included 3 former smokers who stopped smoking 9 months, 3 and 18 years ago respectively. Furthermore, fasting blood samples and periodontal pocket samples for microbiological evaluation were collected. Other clinical procedures have been reported previously (Amaliya et al. 2014).

### *Capacity of vitamin C absorption experiment*

In 2005, 28 subjects were identified showing depleted/deficient plasma vitamin C levels (<4.0 mg/l). All 28 subjects could be retrieved for the present absorption experiment. After the baseline clinical procedures, subjects were instructed to consume the following two days 1 tablet of 60 mg regular vitamin C (ascorbic acid) just before dinner with a glass of water. In the morning after the second tablet again a fasting blood sample was obtained. Thereafter, the subjects followed the same supplementation procedure as the other subjects of the supplementation study.

### *Vitamin C supplementation study*

After the baseline clinical procedures subjects were instructed to consume one tablet a day containing 200 mg Ester C<sup>®</sup> calcium ascorbate, 25 mg calcium threonate and 100 mg citrus flavonoids (vitamin C/Ca/Fl) for 90 days. Tablets were taken with a glass of water just before having dinner. Compliance was assessed by making groups of 6 subjects each who were living in an adjacent place of the village. Each group elected a leader to control the vitamin C intake. Every 30 days, all subjects were asked to bring back the bottle with tablets of which the remaining tablets were calculated.

### *Blood sampling*

At baseline after supplementation fasting venous blood samples were collected into (i) a lithium heparin tube for vitamin C assessment, (ii) an EDTA tube for HbA1c assessment and (iii) a plain tube for CRP analysis, seropositivity of cytomegalovirus (CMV) and Epstein Barr virus (EBV). Tubes were kept at 4°C until analysis in Hasan Sadikin Hospital Bandung-West Java. Plasma for vitamin C analysis was prepared immediately after sampling in order to minimize the oxidation of vitamin C.

### *Microbiological sampling*

Subgingival microbiological samples were taken from the 4 periodontal lesions that were also previously sampled i.e. the deepest bleeding pocket with the greatest amount of attachment loss per quadrant (Timmerman et al. 2001). After careful removal of the supragingival plaque by means of a curette, subgingival plaque samples were taken using 2 sterile paper points per pocket. One paper point was used for bacteriological and the other for viral evaluation. Paper points were suspended in 1ml lysis buffer (Biomérieux, **NucliSens®** Lysis Buffer, Marcy l'Etoile, France) resulting in 2 vials per subject with a pooled sample. Until analysis, vials were kept at 4°C in Hasan Sadikin Hospital Bandung-West Java.

### *Laboratory procedures*

#### *Vitamin C analysis*

After collection, tubes were centrifuged with a low-speed centrifuge (Shanghai Surgical Instrument Factory, Shanghai, China) at 1559xg. for 4 min. to separate plasma from blood cells. To minimize the oxidation of the vitamin C, the latter procedure was performed within 10 min. after sampling. The plasma obtained was subsequently subjected to the preparation procedures according to the instruction manual for Chromsystems High-Pressure Liquid Chromatography (HPLC)-Analysis of Vitamin C in Plasma (Chromsystems, Vitamin C Diagnostics Kit by HPLC, Munich, Germany). Detailed procedures of vitamin C analysis have been reported previously (Amaliya et al. 2007). Plasma vitamin C levels were categorized as follows: normal ( $\geq 4.0$ mg/l), depletion (2 – 3.9mg/l) and deficiency ( $< 2$ mg/l) (Hampl et al. 2004).

#### *HbA1c analysis*

HbA1c analysis was performed by means of Cobas c 501 instrument (Turbidimetric-Inhibition Immunoassay, Roche Diagnostics GmbH, Mannheim, Germany). The anticoagulated whole-blood samples were hemolyzed automatically on the Cobas c 501 analyzer with Cobas c Hemolyzing Reagent Gen.2. Measuring range of HbA1c assessment was 2.3-18.9%, with the lower detection limit of 0.8%. The criteria for HbA1c levels were as follows: normal ( $\leq 5.6\%$ ), pre-diabetes (5.7 – 6.4%), and diabetes ( $\geq 6.5\%$ ) (American Diabetes Association 2012).

*hsCRP analysis*

hsCRP was measured by a particle-enhanced turbidimetric immunoassay on a Cobas c501 instrument (Roche/Hitachi), using reagents from Roche Diagnostics (GmbH, Mannheim, Germany). Measuring range was 0.5-75 mg/l with the lower detection limit of 0.1 mg/l. The classification for CRP levels with regard to risk of cardiovascular disease was as follows: low (<1 mg/l), intermediate (1-3 mg/l), high (> 3 mg/l) (Pearson et al. 2003).

*Seropositivity of CMV and EBV.*

Seropositivity of CMV was measured with the electrochemiluminescence immunoassay ECLIA for the determination of IgG antibodies to CMV in human sera by means of a Cobas c501 immunoassay analyzers (Roche Ltd, Mannheim, Germany). Reagents were purchased from the same vendor and the tests were performed according to the recommendation of the manufacturer. Measuring range was 0.25 – 500 u/ml. Results obtained were interpreted as follows: <6.0 AU/ml = non-reactive and  $\geq$ 6.0 AU/ml = reactive.

Seropositivity of EBV was also assessed by means of an Enzyme Linked Immunosorbent Assay (ELISA) using human antibodies of the IgG against EBV in human serum. Photometric measurement of the colour intensity was made at a wavelength of 450 nm and a reference wavelength of between 620 nm and 650 nm within 30 min. of adding the stop solution. The interpreting results recommended by EuroImmun are as follows: <16 RU/ml = negative,  $\geq$ 16 to <22 RU/ml = borderline,  $\geq$ 22 RU/ml = positive (EUROIMMUN AG, Luebeck, Germany).

*Quantitative polymerase chain reaction (qPCR) for bacterial and viral detection*

Bacterial DNA was extracted and purified using a column system (Spin Protocol, Qiagen, Germany) according to the manufacturer's instructions. Isolated DNA was kept in -80°C until use. Previously published primer/probe sequences and protocol for the bacterial species were used (Bizzarro et al. 2013). In short, quantitative PCR analysis of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythia*, *P. micra*, *F. nucleatum* and *T. denticola*, was carried out with LightCycler®480II (Roche Molecular Diagnostics, Germany).

Viral DNA was isolated using CMV and EBV isolation kit QIAmp DSP Virus and QIAmp DNA Mini Kit (Qiagen Ltd., Hilden, Germany) according to the manufacturer's instructions. Viral DNA were analyzed by means of Light Cycler 2.0™ (Roche Ltd, Penzberg, Germany) with specific reagent for Real-Time PCR artus® Herpes Virus LC-PCR

Kits (Qiagen Ltd., Hilden, Germany). PCR conditions were set according the manufacturer's instructions.

Mean values of bacterial cells and viral copy counts were calculated by dividing the sum of bacterial cells or viral copy counts by the number of subjects that was positive.

### *Statistical analysis*

Descriptive statistics and data analyses were performed with statistical software from SPSS (version 19.0, SPSS Inc., Chicago, IL, USA). Normality testing showed that all variables were non-normally distributed. Therefore, Wilcoxon matched-pairs test was used to analyze data before and after supplementation. Bonferroni corrections were applied for multiple comparisons. Differences were regarded statistically significant at p-values  $\leq 0.05$ .

## Results

### *Capacity of vitamin C absorption experiment*

The evaluation of the baseline plasma vitamin C levels showed that of the 28 subjects that were depleted/deficient in 2005, 15 were again depleted/deficient in the present study. Vitamin C supplementation of 2 days resulted in a significant increase of plasma vitamin C values in all subjects (Table 1). However, of the two deficient subjects one was still deficient after supplementation with vitamin C. In the depleted group of 13 subjects, five remained depleted after supplementation although their plasma vitamin C values increased significantly. This implies that 20.1% of the studied subjects remained depleted/deficient after supplementation with 60 mg vitamin C for two days.

Table 1. Vitamin C (mg/l) values of subjects that showed previously plasma vitamin C levels <4.0 mg/l and took in the present study during 2 days 60 mg/day vitamin C (N=28).

Baseline	2 days after supplementation (N=28)							
	Deficient		Depleted		Normal			
	N	Vitamin C mean (SD)	N	Vitamin C mean (SD)	N	Vitamin C mean (SD)	N	Vitamin C mean (SD)
Deficient	2	1.47 (0.19)	1	1.87	—	—	1	6.90
Depleted	13	2.85 (0.57) * <sup>1</sup>	—	—	5	3.26 (0.48)	8	7.95 (2.53)
Normal	13	7.49 (3.19) * <sup>2</sup>	—	—	—	—	13	10.25 (3.25)

\*significant different from after supplementation(paired tests): \*<sup>1</sup>=0.002, \*<sup>2</sup>=0.002

*Supplementation study*

The mean plasma vitamin C value at baseline of the total group was 5.19 mg/l and included 44 subjects (45%) who showed plasma vitamin C values <4.0 mg/l. After supplementation for 90 days with one tablet vitamin C/Ca/FI per day, in all individual subjects the plasma vitamin C values rose to levels above 4.0 mg/l with a mean of 12.1 mg/l (Table 2), however with a wide range (4.1 to 21.2 mg/l). In 83 subjects plasma vitamin C levels rose to values  $\geq$ 8.8 mg/l. Interestingly, the one with the lowest plasma vitamin C level of 4.1 mg/l after supplementation had also the lowest level before supplementation i.e. 0.5 mg/l.

Table 2. Vitamin C, HbA1c and CRP values of the total group and variable categories before and after supplementation.

Variables assessed in plasma	# subjects before suppl.	Before suppl. mean (SD)	After suppl. mean (SD)	p-value	# subjects in various categories after suppl.
<b>Vitamin C (mg/l)</b>					
Total sample	98	5.19 (3.30)	12.13 (3.35)	0.000	
Normal ( $\geq$ 4.0 mg/l)	54	7.34 (2.97)		0.000	98
Depleted (2.0-3.9 mg/l)	33	2.88 (0.63)	12.29 (3.60)	0.000	
Deficient (<2.0 mg/l)	11	1.48 (0.34)	12.25 (3.60)	0.006	
<b>HbA1c (%)</b>					
Total sample	98	5.96 (0.88)	5.71 (0.66)	0.000	
Normal ( $\leq$ 5.6%)	23	5.42 (0.19)	5.32 (0.18)	0.000	52
Pre-diabetic (5.7-6.4%)	69	5.89 (0.19)	5.70 (0.20)	0.000	44
Diabetes ( $\geq$ 6.5%)	6	8.68 (2.04)	7.28 (2.07)	0.080	2
<b>CRP (mg/l)</b>					
Total sample	98	2.16 (2.73)	0.94 (1.02)	0.000	
Low (<1 mg/l)	45	0.52 (0.21)	0.38 (0.18)	0.000	69
Intermediate (1-3 mg/l)	32	1.69 (0.56)	0.89 (0.48)	0.000	24
High (>3 mg/l)	21	6.36 (3.26)	2.22 (1.50)	0.000	5

All subjects had lower HbA1c levels after supplementation than before. In the total population, the HbA1c levels dropped significantly from 5.96% to 5.71% (Table 2). However, the decrease was less in the subjects that already had normal levels of vitamin C before supplementation (0.10%) compared to the decrease in the pre-diabetic group (0.20%) and the diabetic group (1.40%) ( $p=0.000$ ). It is also interesting to note that after supplementation 41% of the subjects in the pre-diabetic group changed to the normal group and that of the 6 diabetic subjects, 2 remained in the diabetic group whereas 3 changed to the pre-diabetic group and 1 to the normal group.

CRP levels were also lower in all individuals after supplementation than before. In the total group the levels decreased significantly from 2.16 to 0.94 mg/l (Table 2). The decrease for the low, intermediate and high CRP groups was 0.14, 0.80 and 4.14 mg/l respectively, however no statistical significant differences could be assessed between the three groups ( $p=0.12$ ). Interestingly, 66% of the intermediate CRP group changed to the low group, and of the high CRP group, 62% of individuals changed into the intermediate group and 14% to the low group. Explorative analysis showed that in smokers the decrease of CRP levels was significantly less than in non-smokers ( $p=0.031$ ).

The microbiological results of vitamin C/Ca/FI supplementation are presented in Table 3. It can be seen that at baseline almost all subjects were positive for the studied putative periodontopathic bacteria, with exception of *A. actinomycetemcomitans* which was present in almost half of the population. The supplementation had no effect on the occurrence of the bacteria, however for all bacteria a significant decrease in their quantity was found after supplementation.

Analysis of serum showed that all subjects were sero-positive for EBV and CMV. Subgingival results showed that at baseline CMV was not detected in any of the subjects, whereas in 75% of the subjects EBV could be detected. After supplementation the latter dropped to 35% of subjects with a significantly lower number of copy counts (Table 3).

Table 3. Subgingival prevalence of microorganisms before and after vitamin C supplementation; number of positive subjects and mean number of bacterial cells ( $\times 10^3$ ) or mean number of viral copy counts per ml ( $\times 10^6$ ) in positive subjects (total number of subjects is 98).

Microorganisms	# positive subjects		Mean value (SD)		p-value
	Before	After	Before	After	
<i>A. actinomycetemcomitans</i>	46	45	1.70 (3.89)	1.25 (4.96)	0.014
<i>P. gingivalis</i>	97	97	24.84 (32.35)	7.77 (9.23)	0.000
<i>P. intermedia</i>	98	98	0.32 (0.39)	0.13 (0.14)	0.000
<i>T. forsythia</i>	98	98	1.23 (7.65)	0.14 (0.16)	0.000
<i>P. micros</i>	98	98	0.75 (0.84)	0.38 (0.37)	0.000
<i>F. nucleatum</i>	98	98	0.10 (0.32)	0.04 (0.07)	0.000
<i>T. denticola</i>	98	98	1.66 (2.14)	0.75 (1.10)	0.000
Epstein-Barr virus (EBV)	73	34	20.69 (57.33)	16.36 (91.16)	0.000
Cytomegalovirus (CMV)	0	4	0	0.29 (0.11)	0.612

## Discussion

The results of the absorption experiment showed that 60 mg vitamin C supplementation does not result for all individuals in normal plasma vitamin C levels, i.e.  $\geq 4.0$  mg/l. This phenomenon has been previously shown by Levine et al. (1996) in a study in which steady-state plasma concentrations were determined at seven daily doses of vitamin C from 30 to 2500 mg. Especially at 60 mg, large variations were found between subjects, ranging from 2.6-10.3 mg/l. These discrepancies between subjects can be explained by genetic variations in vitamin C transporter protein SVCT1 that can influence plasma vitamin C concentrations (Cahill & El-Sohemy 2009, De Jong et al. 2014). In the subgroup of 28 subjects, 6 (20%) remained depleted/deficient after supplementation for two days with 60 mg vitamin C. Since these subjects are inhabitants of the same village and the whole cohort was only selected on the basis of their age in 1987, it may be supposed that this figure of 20% is also applicable to the total study population.

In the present supplementation experiment a commercially available vitamin C supplement (vitamin C/Ca/Fl) was chosen that contained 200 mg Ester C<sup>®</sup> calcium ascorbate but also 100 mg citrus flavonoids. As vitamin C supplement, Ester C<sup>®</sup> was selected since, due to its chemical structure, it has a neutral pH value and compared with ascorbic acid (vitamin C) causes significantly fewer epigastric adverse effects in subjects sensitive to acidic foods (Gruenwald et al. 2006). The choice for a product that includes citrus flavonoids was motivated in order to make the supplement similar to fruit. Citrus flavonoids have anti-inflammatory properties due to the inhibition of the synthesis and biological activities of different pro-inflammatory mediators (Benavente-García & Castillo 2008). In addition, like vitamin C, citrus flavonoids are powerful antioxidants against free radicals due to their hydrogen-donating ability (Tripoli et al. 2007). The choice for the vitamin C dose of 200 mg Ester C<sup>®</sup> was based on the assumption that with this dose in all subjects optimal plasma values  $\geq 8.8$  mg/l would be obtained as suggested by Levine et al. (1996) and Gey (1998). Nevertheless, this value was not achieved in 15 out of the total 98 subjects although for all normal values above 4.0 mg/l were achieved.

The supplementation with vitamin C/Ca/Fl had major impact on the HbA1c values, especially in the pre-diabetic subjects of which 41% had normal HbA1c values after supplementation instead of 71% before. The effect was even greater in the 6 diabetic subjects of which only 2 remained, based on HbA1c values, in the diabetic group. This effect on HbA1c levels can be contributed to the vitamin C in the supplement as well as to the citrus flavonoids which are both present in citrus fruit. It has been shown that an inverse association

exists between plasma vitamin C and HbA1c (Sargeant et al. 2000, Kositsawat & Freeman 2011). Prospective studies showed that higher plasma vitamin C levels and, to a lesser degree, fruit and vegetable intake were associated with a substantially decreased risk of diabetes (Harding et al. 2008). Recently it was reported that diets rich in flavonoids are associated with a lower incidence of type 2 diabetes (Zamora-Ros et al. 2013). This phenomenon may be explained by a reduced glucose uptake (Li et al. 2006) and the flavonoids capacity of slowing or preventing the oxidation of other molecules (Landete 2012). Cooper et al. (2012) found also that fruit and vegetables intake was inversely related to the risk of diabetes, however when analyzed separately this association was no longer present. Vitamin C supplement usage by individuals has been shown to be associated with a significantly lower risk of diabetes (Song et al. 2011). Supplementation of diabetics with high amounts of vitamin C (500-1000 mg/day) resulted in a decrease of HbA1c values (Vinson & Howard 1996, Afkhami-Ardekani & Shojaoddiny-Ardekani 2007, Dakhale et al. 2011). Recently it was shown that supplementation of lower doses vitamin C (200 mg/day) decreases also HbA1c levels in diabetics (Mahmoudabadi et al. 2011). This latter finding corroborates well with the findings of the present study. The most likely explanation for the decrease in HbA1c after supplementation with vitamin C is the competition of vitamin C with glucose for reaction with protein amino groups (Davie et al. 1992).

The CRP levels decreased also markedly after supplementation and the number of subjects with low risk for cardiovascular disease, based on CRP levels, increased from 46% before to 70% after supplementation. Like for HbA1c, this can be explained both by the vitamin C as well as by the citrus flavonoids in the supplement. Inverse relationships has been shown between plasma vitamin C levels and CRP (Ford et al. 2003, Wannamethee et al. 2006, Mah et al. 2011). An inverse relationship was found also between fruit intake, dietary vitamin C intake and CRP levels (Wannamethee et al. 2006, Floegel et al. 2011). Notably, it has been shown that 1000 mg vitamin C supplementation resulted in a significant decrease of CRP values (Block et al. 2009). The effect of citrus flavonoids alone on CRP is difficult to evaluate due to the confounding presence of vitamin C in most studies. However, Askari et al. (2012) found a trend towards lower CRP levels after two months of quercetin supplementation (a flavonoid).

A recent periodontal study showed that 450 mg vitamin C supplementation without concomitant periodontal treatment resulted in subjects with gingivitis in a decrease of bleeding whereas in periodontitis patients no effect was found (Gokhale et al. 2013). Since the present study included an untreated periodontitis population no attempt was made to

evaluate possible effects on the periodontal condition and choose for only evaluation the subgingival microflora. Surprisingly, we found for all studied bacteria and EBV that the number of organisms were significantly lower after supplementation. As far as we know, such an effect of vitamin C has never been shown before but supports the view that in periodontal treatment evaluation of the vitamin C status may be warranted.

A weakness of the present study is that, due to the decision of the Ethic Committee, it is an observational study and not a randomized controlled trial. However, since in all subjects the HbA1c and CRP levels were lower after supplementation we believe that the chance for a false positive results may be negligible. The present results should be considered in the light of the relatively poor diet of this population, consisting mainly of three servings of white rice per day (Amaliya et al. 2014).

In conclusion, in populations with poor dietary conditions emphasis should be paid by the authorities to improve the fruit intake.

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